

The Compatible Solute Ectoine Protects against Nanoparticle-induced Neutrophilic Lung Inflammation

Ulrich Sydlik¹, Inka Gallitz¹, Catrin Albrecht¹, Josef Abel¹, Jean Krutmann^{1*}, and Klaus Unfried^{1*}

¹Environmental Health Research Institute (IUF) at the Heinrich-Heine-University Düsseldorf gGmbH, Düsseldorf, Germany

Rationale: Inflammatory reactions of the airways induced by nanoparticles of occupational and environmental origin contribute to organ-specific and systemic human diseases. Because this kind of exposure in modern societies is often unavoidable, a strategy of molecular prevention on an individual level could help to prevent inflammation-derived secondary diseases.

Objectives: To test whether the compatible solute ectoine [(S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid], which is known to reduce cell stress effects on a molecular level, prevents nanoparticle-induced lung inflammation.

Methods: Inflammatory parameters were studied in Fischer 344 rats treated with model carbon nanoparticles. The molecular effects of ectoine on proinflammatory signal transduction were demonstrated in the rat and in the human system using cultured lung epithelial cells.

Measurements and Main Results: Ectoine, given with or before the nanoparticles, dose-dependently reduced neutrophil inflammation in the lung. This preventive effect was not observed when lung inflammation was induced by bacterial lipopolysaccharide. Analyses of the underlying mode of action revealed that ectoine acted on lung epithelial cells. Ectoine administration inhibited nanoparticle-induced signaling, which is known to be responsible for proinflammatory reactions in rat lung epithelial cells *in vitro* as well as *in vivo*. These findings were corroborated and extended in experiments with cultured human bronchial epithelial cells in which ectoine inhibited nanoparticle-triggered cell signaling and IL-8 induction. **Conclusions:** Because compatible solutes are compliant natural products without known toxic potential, we propose that this group of substances may be used for the prevention of particle-induced airway inflammation in humans.

Keywords: lung epithelium; mitogen-activated protein kinases; IL-8

Inflammation of the airways is a central pathogenic mechanism responsible for a multitude of organic and systemic diseases. Besides biogenic triggers, lung inflammation can be induced by chronic inhalation of particulate xenobiotics coming from natural and man-made environmental sources. Among the plethora of potentially inhaled particles, ultrafine or nanoparticles of occupational or environmental origin have been identified as major driving hazards in the ambient air of modern industrialized societies (1). The lung, as the primary target organ for particle-induced inflammation, is severely impaired by the constant recruitment and activation of inflammatory cells. Lung diseases like emphysema, chronic obstructive pulmonary disease, fibrosis,

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Compatible solutes are considered as stabilizers of macromolecules, including membrane structures. There are few indications that this group of substances could be used for the prevention or therapy of diseases.

What This Study Adds to the Field

Ectoine applied to the lung prevents neutrophil inflammation induced by environmentally relevant model nanoparticles.

and cancer (2–4) as well as systemic disorders on the level of the cardiovascular and the immune system (5, 6) occur as a consequence of these events.

Lung inflammation is thus an obvious target for the prevention of particle-induced diseases. Particle pollution in ambient air can be reduced but not eliminated, and conventional measures will therefore always be of limited effectiveness at an individual level. Thus, there is a growing need for the development of individualized preventive strategies that can be used by susceptible persons, such as children and elderly persons, or by patients with preexisting disorders, such as asthma or cardiovascular disease.

Such a preventive strategy should make use of compounds that are well tolerated and have few or no side effects. The reduction of cellular stress by compatible solutes (CS) is an evolutionary highly conserved biological principle that is used in organisms from bacteria up to higher vertebrates (7). CS are small, organic, mostly neutral or zwitter-ionic cytoprotectants that are produced by cells under stress conditions. Little is known about the use of CS for the treatment or prevention of lung diseases, but the few existing studies indicate that CS affect signal transduction processes that are relevant for lung fibrosis (8–10). It is not known whether CS can be used for the prevention or treatment of lung inflammation.

In the present study, we assessed the antiinflammatory effect of the CS ectoine *in vivo* in rats and in a human cell culture system. Lung inflammation was induced by carbon nanoparticles (CNPs), which are well-accepted model particles representing the carbonaceous core of combustion-derived nanoparticles (1). Ectoine, a cyclic amino acid from halophilic bacteria (11), was chosen because (1) it can be produced in an industrial scale with high quality standards (12), (2) no toxic side effects of ectoine are known (13), (3) ectoine was previously shown to protect human skin epithelial cells against UV radiation (14), and (4) these photoprotective effects are based upon the capacity of ectoine to interfere with signaling pathways that are initiated at the level of the cell membrane (15). We recently observed that molecular effects of inhalable nanoparticles in human and rat lung epithelial cells are mediated by membrane receptor-dependent signaling pathways (16, 17). The nanoparticle-specific activation of the

(Received in original form December 19, 2008; accepted in final form March 25, 2009)

* These authors contributed equally to this work.

Supported by Zukunftswettbewerb Ruhrgebiet (project no. 005-0507-0018), Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit, Deutsche Forschungsgemeinschaft (grants SFB 728, GK 1033, and GK 1427).

Correspondence and requests for reprints should be addressed to Dr. Klaus Unfried, Ph.D., Institut für Umweltmedizinische Forschung (IUF), Auf'm Hennekamp 50, 40225 Düsseldorf, Germany. E-mail: klaus.unfried@uni-duesseldorf.de

Am J Respir Crit Care Med Vol 180, pp 29–35, 2009

Originally Published in Press as DOI: 10.1164/rccm.200812-1911OC on March 26, 2009
Internet address: www.atsjournals.org

mitogen-activated protein kinases (MAPKs) ERK1/2 (extracellular regulated kinases 1 and 2) in lung epithelial cells is strongly dependent on the cooperative activation of the membrane receptors epidermal growth factor receptor and β_1 -integrins. A stabilization of membrane structures accomplished by a compatible solute such as ectoine could therefore lead to a reduction of CNP-induced adverse effects in the lung. The hypothesis that such an effect is due to the stabilizing properties of the compound was tested by the comparison of the compatible solutes ectoine and di-myoinositol-phosphate (DIP) with a nonstabilizing compound (urea).

METHODS

Particle Suspensions and Compound Solutions

CNPs (14 nm in diameter) (Carbon Black, Printex 90; Degussa, Frankfurt, Germany) were suspended in PBS by sonication (1-ml aliquots, 120 W, 50–60 Hz) for at least 1 hour or up to immediate usage for further dilution or instillation. Ectoine [(S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid, lipopolysaccharide (LPS)-free, ultrapure 99%; bitop AG, Witten, Germany] was solubilized in PBS (440 $\mu\text{g/kg}$ body weight = 0.4 ml of a 1 mM solution and the indicated dilutions). DIP (LL-di-myoinositol-1,1'-phosphate, purity $\geq 90\%$; bitop AG) and urea (purity $\geq 99.5\%$, Fluka/Sigma-Aldrich, Taufkirchen, Germany) were treated similarly.

Animal and Cell Culture Experiments

Female Fisher 344 rats (8-week-old; Charles River Laboratories, Sulzfeld, Germany) were instilled intratracheally with a volume of 0.4-ml suspension (doses indicated in the figures) under inhalation anesthesia (isoflurane, 5%, 2 minutes). Animals were killed by exsanguination under pentobarbital anesthesia 48 hours after CNP (or control) treatment. Lung lavages were performed using 4×5 ml PBS. Differential cell counts were performed from Giemsa/May-Grünwald stainings of lavage cells. Cell-free lavage fluids were used for cytokine assays. Lung tissues were minced, shock frozen, and stored at -80°C until further use. Immunosections were performed as described (18). All animal experiments were performed after relevant permission according to German animal protection laws.

Cell culture and *in vitro* experiments with 16HBE14o⁺ and RLE-6TN were performed as described (17). CS were applied 4 hours before particle treatment. MAPK phosphorylation was determined 1 hour (P38) and 8 hours (ERK1/2) after CNP treatment. Preparation of mRNA was performed 4 hours after CNP application.

Cytokine and MAPK Assays

Cytokine levels were detected by solid-phase ELISA (Cinc-1; R&D systems, Minneapolis, MN) or a rat cytokine antibody arrays (RayBio-tech, Inc., Norcross, CA) according to the manufacturers' instructions. Signal strength was determined densitometrically from autoradiographs using Quantity One software (version 4.1; Bio-Rad, Hercules, CA).

Western blots and the respective protein preparations were performed as described (16). The following antibodies were used: total ERK1/2 (human and rat), p44/42 MAP kinase antibody (Cell Signaling Technology, Danvers, MA); phospho-ERK1/2 (human), rabbit (polyclonal) anti-ERK1/2 [pTyr^{185/187}] phosphospecific antibody (Bio-source, Belgium); phospho-ERK1/2 (rat), phospho-p44/p42 MAP Kinase (Thr202/Tyr204) antibody (Cell Signaling Technology); total-P38 (human and rat), P38 MAP Kinase antibody (Cell Signaling Technology); phospho-P38 (human and rat), Phospho-p38 MAP Kinase (Thr180/Tyr182) antibody (Cell Signaling Technology).

Immunohistochemistry

Phosphorylated MAPK were detected as described (18) using the following antibodies: phospho-p44/p42 MAP Kinase (Thr202/Tyr204) and Phospho-p38 MAPK (Thr180/Tyr182, 12F8) (both from Cell Signaling Technology).

Quantitative RT-PCR

IL-8 mRNA was determined by RT-PCR using Lightcycler technology (Roche, Germany) according to the respective manuals. As house-

keeping control, separate runs with 18S-rRNA were performed. Primers were IL-8fw 5'-ACTCCAAACCTTTCACCC-3', IL-8rev 5'-CCCTCTTCAAAAACCTCTCCAC-3', 18Sfw 5'-GCCGCTAGA GGTGAAATTCCTG-3, and 18Srev 5'-CATTCTTGCAAATGCT TCG-3'.

Statistics

All animal data represent the mean values of five or more animals per group (higher animal numbers are indicated). For *in vitro* assays, at least three independent experiments were performed. Data show mean values and standard errors. Significance was calculated from original

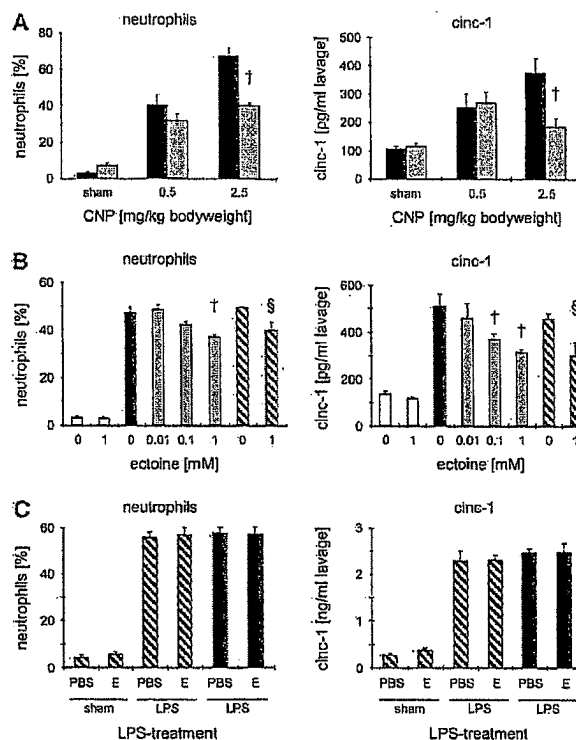


Figure 1. Ectoine reduces dose-dependently carbon nanoparticle (CNP)- but not lipopolysaccharide (LPS)-induced inflammation *in vivo*. Bronchoalveolar lavage parameters (% neutrophils and cinc-1) from Fischer 344 rats intratracheally instilled with proinflammatory agents (CNP and LPS, each 0.4 ml) in the presence or absence of ectoine are shown. (A) Ectoine effects on different doses of CNP after 48 hours. Animals ($n = 5$) were instilled once with phosphate-buffered saline (PBS) (sham) or with 0.5 or 2.5 mg/kg body weight CNP alone (solid bars). Shaded bars indicate an instillation of CNP together with 1 mM ectoine. (B) Dose dependency and pretreatment with ectoine. Animals were instilled once (solid bars) with 2.5 mg/kg body weight CNP in the absence (solid bar) or in the presence of the indicated concentrations of ectoine (shaded bars). Hatched bars: Animals were pretreated twice (-96 and -48 hours) with PBS (0) or with 1 mM ectoine (1) before CNP treatment (2.5 mg/kg body weight). Open bars indicate control treatment ($n = 5$ for the control group; $n = 6$ to 8 in the treatment groups). The parameters were determined 48 hours after CNP application. (C) Ectoine effects on LPS treatment after 4 hours. Animals were pretreated (hatched bars; -96 and -48 hours) twice with PBS or 1 mM ectoine (E) before the application of 2 $\mu\text{g/kg}$ body weight LPS. Solid bars: Animals were instilled once with 2 $\mu\text{g/kg}$ body weight LPS together with PBS or 1 mM ectoine. *Significantly different from CNP treatment without ectoine. †Significantly different from PBS pretreatments.

data with Student's *t* test using SPSS software version 12.01 (SPSS Inc., Chicago, IL).

RESULTS

Ectoine Effects on CNP-induced Neutrophilic Inflammation

Inhalation of chemically inert particles with dimensions in the nanometer range is a well-known cell stress leading to inflammatory reactions of the lung (1). For mechanistic studies of this phenomenon, intratracheal instillation of suspended particles in rats is a well-evaluated and accepted experimental system (1). Accordingly, in the present study, 0.5 and 2.5 mg/kg body weight CNPs instilled intratracheally in rats triggered a strong pulmonary inflammation. The total cell number in the bronchoalveolar lavage (BAL) of the higher dosage group 48 hours postinstillation was $1.50 \times 10^5 \pm 0.26 \times 10^5$ cells/ml, compared with $0.75 \times 10^5 \pm 0.18 \times 10^5$ in the control-treated animals (PBS). This increase in cell number resulted from an influx of neutrophilic granulocytes (Figure 1A). The numbers of other inflammatory cells (monocytes/macrophages, eosinophilic granulocytes, and lymphocytes) were not changed. The CNP-induced neutrophil influx was significantly reduced if the instilled particle suspension contained 1 mM ectoine (Figure 1A). Similarly, ectoine also prevented the CNP-induced increase in cinc-1 release, determined by ELISA in the lavage fluid. This cytokine is the rat homolog of IL-8 and mediates the CNP-induced influx of neutrophilic granulocytes in this model (19).

In a second, independent experiment (Figure 1B), a broad dose range from 0.01–1 mM of ectoine was tested. For both CNP-induced endpoints (i.e., neutrophils and cinc-1), dose-dependent protective effects were observed, which proved to be statistically significant for neutrophils in the highest dose and for cinc-1 release for 0.1 and 1 mM ectoine.

The presence of ectoine in the sample together with CNPs could theoretically have an effect on the physico-chemical properties of the nanoparticles and thereby lead to an artifact that would account for the observed loss in proinflammatory activity of the nanoparticle suspension. In this regard, we have observed that ectoine concentrations in solution did not decrease in the presence of CNPs, indicating that ectoine was not absorbed onto the surface of the nanoparticles (data not shown). More importantly in this context, the ectoine effect was detectable to similar extents if the CS (1 mM) was instilled twice (i.e., 96 and 48 hours before CNP instillation) (Figure 1B). Therefore, these data demonstrate that the preventive effect of ectoine is not an artifact of unspecific nanoparticle modification by this compound.

Ideally, prevention of lung inflammation would be restricted to particle-induced inflammation and would not compromise inflammatory reactions induced by infectious agents. We therefore assessed the effect of ectoine administration in a model of bacterial pathogen-induced lung in-

flammation. The bacterial molecule LPS induces cell responses by specific receptor ligand binding (i.e., a mechanism completely different from the cellular stress induced by nanoparticles) (20). LPS, at a dose of 2 μ g/kg bodyweight 4 hours postinstillation, induced an inflammatory response on the level of neutrophils that was of comparable strength to the particle treatment after 48 hours (Figure 1C). The cytokine cinc-1 exhibited considerably higher levels in BAL of LPS-treated animals compared with CNP-treated animals. This difference may be considered as indicative for the different mechanisms eliciting inflammation by LPS and CNPs. Under the chosen conditions, ectoine was not able to reduce the kind of inflammatory reaction induced by LPS, regardless of whether the CS was administered before or together with the bacterial component. Cinc-1 levels remained unchanged by the ectoine treatment.

Cytokine Profiles Modulated by Ectoine

Inflammatory cytokines induced by CNPs are suspected to be responsible for secondary or systemic diseases. Therefore, in the present study, cytokine arrays from lavage fluids from control animals and CNP-exposed animals with or without ectoine pretreatment (–96 and –48 hours; see Figure 1B) were analyzed. Almost all inflammatory cytokines that showed elevated levels 48 hours after CNP instillation were significantly reduced by ectoine (Figure 2). At the chosen time point, ectoine pretreatment had no effect on IFN- γ and IL-10 levels in BAL.

MAPK Activity in Lung Tissue

The previous experiments indicate that ectoine is able to interfere with inflammatory reactions of the lung induced by CNPs. For nanoparticle-induced effects, the lung epithelium, with its large surface, is considered as a main target of particle tissue interaction (1). In this tissue, inflammatory reactions can be triggered by an activation of MAPK (21). To test the hypothesis that ectoine prevented nanoparticle-induced lung inflammation by inhibiting these signaling pathways, we first measured phosphorylation of ERK1/2 and P38 in homogenates of whole lungs isolated from CNP- and ectoine-treated rats. Administration of 1 mM ectoine significantly decreased CNP-induced phosphorylation of MAPK in the lung tissue of rats (Figures 3A and 3C). This observation was corroborated and extended by immunostaining of lung sections, demonstrating that activation of these MAPK by CNPs was reduced in the alveolar epithelial regions upon ectoine administration (Figures 3B and 3D). This preventive effect of ectoine was clearly visible just in the epithelium. Although not tested quantitatively, in macrophages that showed also a strong staining for activated MAPK after CNP treatment, and to some extent also in control animals (Figure 3B, PBS), such an effect was not obvious.

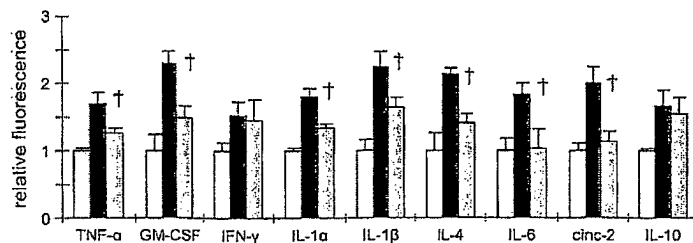


Figure 2. Cytokine profiles modulated by ectoine. Cytokine patterns in bronchoalveolar lavage (BAL) from control animals (open bars) and animals pretreated twice with phosphate buffered saline (solid bars) or 1 mM ectoine (shaded bars) before the application of 2.5 mg/kg body weight carbon nanoparticles (see Figure 1B). Cytokine levels from groups shown in Figure 1B were determined by membrane-coupled cytokine arrays. All cytokine levels from animals treated with particles alone were significantly increased compared with control animals. *Significantly different from carbon nanoparticle + phosphate-buffered saline pretreatments.

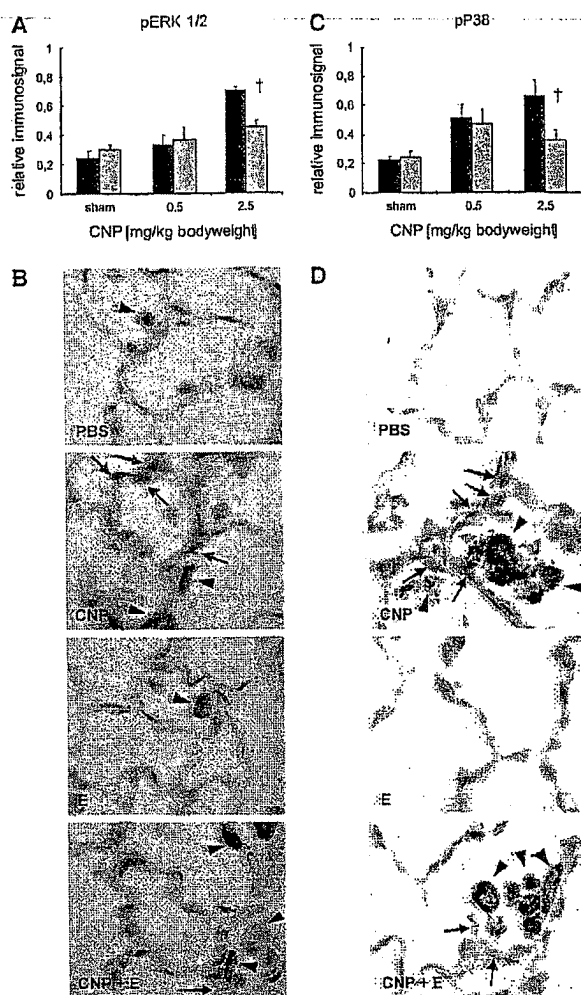


Figure 3. Prevention of carbon nanoparticle (CNP)-dependent mitogen-activated protein kinase (MAPK) activation *in vivo* in epithelial cells. MAPK activation in lungs of animals treated as described in Figure 1A determined by Western Blot and immunohistochemistry is shown. (A) Phospho-ERK 1/2 signals in whole lung tissue relative to total ERK 1/2 signals determined by Western blot analyses. (B) Immunohistochemistry of phosphorylated ERK 1/2 in lung sections from animals treated as described in Figure 1A (phosphate buffered saline [PBS], control; CNP, 2.5 mg/kg bodyweight; E, 1 mM ectoine). (C) Phospho-P38 signals in whole lung tissue relative to total P38 signals determined by Western Blot analyses. (D) Immunohistochemistry of phosphorylated P38 in lung sections from animals treated as described in Figure 1A (PBS, control; CNP, 2.5 mg/kg bodyweight; E, 1 mM ectoine). (A and C) Solid bars: Animals treated with the indicated doses of CNPs or PBS (sham). Shaded bars: Respective treatments in the presence of 1 mM ectoine. *Significantly different from CNP treatment alone. (B and D) Counterstaining with hematoxylin-eosin (magnification $\times 1,000$). Positively stained cells (arrows, epithelial cells; arrowheads, macrophages) are indicated. The black staining in macrophages is considered as agglomerated CNPs.

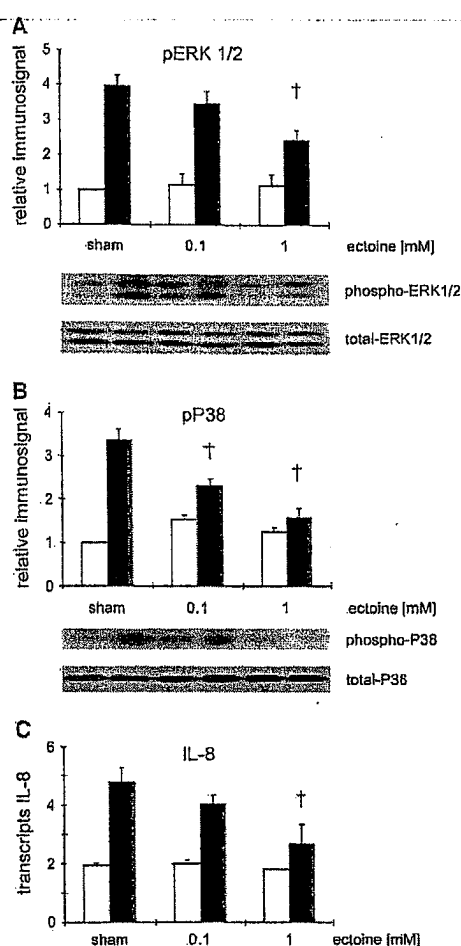


Figure 4. Ectoine effects in human bronchial epithelial cells *in vitro*. Activation of mitogen-activated protein kinase and up-regulation of IL-8 mRNA in 16HBE14o⁻ cells. Quantification and representative immunoblots of (A) phospho-ERK 1/2 and total ERK 1/2 and (B) phospho-P38 and total-P38 in cells treated with phosphate buffered saline (PBS) (open bars) or 10 $\mu\text{g}/\text{cm}^2$ carbon nanoparticle (CNP) (solid bars) with the indicated doses of ectoine. Immunosignals are given as relative increases compared with PBS-treated control cells. (C) IL-8 mRNA in cells treated with 10 $\mu\text{g}/\text{cm}^2$ CNPs with or without 0.1 or 1 mM ectoine. *Significantly different from CNP treatment alone.

Ectoine Effects on Human Bronchial Epithelial Cells

MAPK activation has been described as a direct consequence of nanoparticle cell interaction (16, 21). The interference of ectoine with this molecular process was tested in an *in vitro* system using a human bronchial epithelial cell line (16HBE14o⁻) (22). Treatment of the cells with a noncytotoxic dose (17) of 10 $\mu\text{g}/\text{cm}^2$ CNPs resulted in significant increases of ERK1/2 activation 8 hours after treatment and P38 activation 1 hours after treatment (Figures 4A and 4B). Accordingly, IL-8 mRNA was up-regulated 4 hours after particle application (Figure 4C). Similar to the rat *in vivo* data, pretreatment with ectoine (4 hours before CNP) resulted in a dose-dependent reduction of MAPK activation and IL-8 expression, which was statistically significant for P38 at both ectoine concentrations (0.1 and 1 mM) and for ERK1/2 and IL-8 at the higher dose.

Effect of CS Compared with Another Solute

CS, unlike inorganic solutes, do not perturb normal cell functions and are therefore highly compliant. To test this concept, additional experiments including CS and non-CS compounds were performed. DIP is a CS from thermophilic bacteria with well-known stabilizing properties (23). In contrast, the osmolyte urea, because of its direct interaction with charged surfaces of macromolecules, is thought to have a negative impact on the hydration layer and is therefore not considered as a CS (7). The application of the test substances (1 mM) together with CNPs in rats revealed a significant reduction of the inflammatory reaction on the level of neutrophils and on cinc-1 release in BAL only for ectoine and DIP (Figure 5A). Urea in this dose range had no effect on these inflammatory parameters. Similar differences were observed in *in vitro* experiments on the level of MAPK activation. Phosphorylation of the MAPK ERK1/2 and P38 was studied *in vitro* using a rat alveolar type II cell line (RLE-6TN) (24). The exposure scenario was identical to that previously used in the human system (Figure 4). CNP-induced MAPK activation was also inhibited by the pretreatment of cell with ectoine or DIP (0.01 and 0.1 mM), whereas urea at doses comparable to the CS had no effect on this cellular stress response (Figure 5B).

DISCUSSION

Ectoine Significantly Reduces Nanoparticle-Induced Neutrophilic Inflammation

Exposure of humans to inhaled nanoparticles is associated with a number of detrimental consequences that extend beyond lung inflammation and include secondary diseases such as asthma and chronic obstructive pulmonary disease as well as systemic diseases like coronary heart disease (1). The importance of neutrophilic inflammation as a response against this environmental stress has been documented in studies with healthy volunteers inhaling environmentally relevant concentrations of diesel exhaust particles (25). Bronchial washes 18 hours after a 2-hour exposure showed a 1.7-fold increase in neutrophils induced by this treatment, whereas the numbers of all other cell types remained unaffected. In the animal experiments described here, we were able to induce a much stronger (20-fold) inflammatory response, which was also exclusively driven by neutrophil influx.

Under these conditions, the application of 1 mM ectoine together with the nanoparticles significantly reduced lung inflammation, suggesting that the mild chronic inflammation induced by environmental pollutants in humans may be prevented by ectoine.

The preventive effect of ectoine proved to be dose dependent and could also be observed if ectoine was applied before CNP application, indicating the specificity of this phenomenon for the CS treatment. These results also show that the ectoine effect is not due to an interaction of the CS with the particles, which might reduce the reactive surface area and thereby diminish the proinflammatory effects. Instead, prevention of CNP-induced lung inflammation represents an ectoine-dependent reaction of the organism.

Our results suggest testing ectoine as a protectant against unavoidable environmental, nanoparticle-induced stress in human airways. In this context, however, essential mechanisms of innate immunity should not be impaired. We therefore, as a first approach to address this question, tested the effects of ectoine on LPS-triggered neutrophil recruitment. Although we cannot exclude that ectoine treatment has an effect on LPS-induced inflammation at later time points, our experiments suggest that the immediate early response against bacterial pathogens is not impaired by this CS. Because this important defense mechanism is apparently not compromised, it is likely that prevention of particle-induced lung inflammation by ectoine administration will have little or no negative impact on host defense mechanisms of the lung.

Inflammation-derived diseases are proposed to be partially induced or modulated by proinflammatory cytokines, which may trigger secondary pathogenic events and translocate into the circulation and thereby cause systemic effects. In our experiments, besides IFN- γ and IL-10, all CNP-induced proinflammatory cytokines were reduced in animals pretreated with ectoine. In particular, CNP-induced cytokine levels that were decreased upon ectoine instillation included TNF- α , which plays a critical role in the induction of lung fibrosis (26), as well as several interleukins, such as IL-4 and IL-6, which are well known for their role in the pathogenic cascade leading to asthma (6). In the lung, IFN- γ is considered to serve as an activator of macrophages necessary for proper bacterial pathogen defense (27), and IL-10 has been shown to serve as an autoregulatory factor that reduces IL-8 production in neutrophils (28). These results, on one hand, reflect the general antiinflammatory response of the lung to the

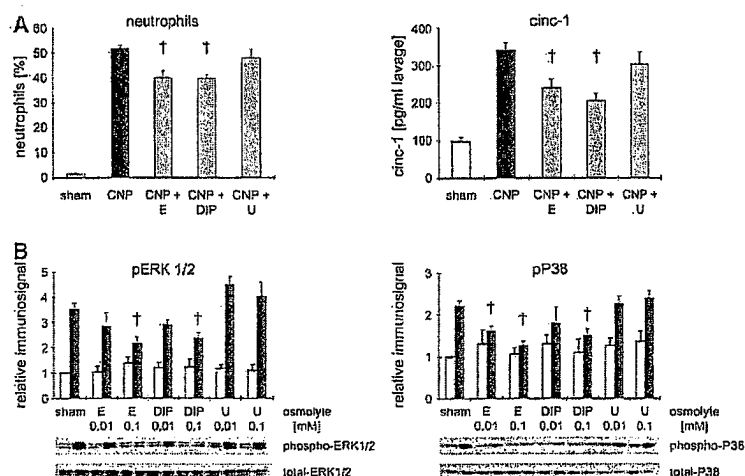


Figure 5. Compatible solutes (CS) but not urea prevent neutrophil inflammation *in vivo* and the respective signaling *in vitro*. (A) Bronchoalveolar lavage parameters (% neutrophils and cinc-1) from Fischer 344 rats intratracheally instilled with carbon nanoparticles (CNPs) in the presence or absence of the test substances ectoine (E), di-myoinositol-phosphate (DIP), or urea (U) 48 hours after CNP instillation. Animals ($n = 5$) were instilled once with phosphate buffered saline (PBS) or 2.5 mg/kg body weight CNP alone (solid bars). Shaded bars indicate an instillation of the same CNP dose together with 1 mM test substance. (B) Activation of mitogen-activated protein kinase in RLE-6TN cells. Quantification and representative immunoblots of phospho-ERK1/2 and total ERK1/2 as well as phospho-P38 and total-P38 in cells treated with PBS (open bars) or 10 $\mu\text{g}/\text{cm}^2$ CNP (solid bars) with the indicated doses of test substances. Immunosignals are given as relative increases compared with PBS-treated control cells. *Significantly different from CNP treatment alone.

ectoine treatment. On the other hand, they suggest a possible preventive effect against secondary diseases induced or modulated by neutrophilic inflammation.

Ectoine Acts on MAPK Activation in Lung Epithelial Cells

We previously described the activation of MAPK ERK1/2 as a nanoparticle-specific reaction of lung epithelial cells that is not elicited by particles of bigger size classes. This intracellular signaling pathway, which is initiated at the level of cell surface receptors, was specific for nanoparticle-induced epithelial cell proliferation (16). CNP-specific up-regulation of IL-8 as the predominant cytokine driving neutrophil influx in lung epithelial cells, however, has been demonstrated to be strictly dependent on the activation of the MAPK P38 (29). Investigations of nanoparticle-driven NF- κ B activation revealed that this cellular stress response is of minor importance and, therefore, emphasized the central role of MAPK in nanoparticle-induced lung inflammation in this tissue (29). Both MAPK signaling pathways have been shown to be directly induced by nanoparticle cell interaction. In the current study, the CNP-specific activation of ERK1/2 and P38 was prevented by ectoine *in vivo* as well as *in vitro* in human bronchial epithelial cells and in rat alveolar epithelial cells. These findings are consistent with a direct impact of ectoine on nanoparticle cell interaction in the lung epithelium. Although the effect of ectoine on inflammatory cells was not quantified in our experiments, the immunostaining of both MAPKs indicate the central role of epithelial cells in nanoparticle-induced lung inflammation as well as in the preventive effect of ectoine.

Relevance for Humans

Because CS are compliant natural materials without known toxic potential, we propose that ectoine can be used for the development of preventive medical strategies to protect susceptible individuals against nanoparticle-induced lung inflammation. In this context, the finding that in human bronchial epithelial cells P38 activation as well as IL-8 expression can be reduced almost to control levels by pretreatment of the cells with 1 mM ectoine appears very promising. Because the CNPs used in this study are considered as well-defined model particles for environmental combustion-generated nanoparticles (e.g., diesel exhaust particles), experiments using such real-life particles will be of particular interest. Moreover, the systemic application of CS, which may be more convenient compared with aspiration, could be tested.

Molecular Mechanisms of CS Effects

The precise mechanism by which ectoine inhibits CNP-induced signaling is not known. One property of CS is that they act as osmolytes in the regulation of cellular hydration (7). Even more important in the context of this study is the fact that CS serve as cytoprotectants under various stress conditions. Accordingly, in the presence of CS, compact protein structures are thermodynamically preferred conformations (30), and, during protein synthesis, CS act as chemical chaperones helping the growing molecule to fold in a proper manner (31). These stabilizing features are used for biotechnological applications (32), and there is evidence that CS may be used as therapeutic or preventive agents stabilizing organ functions in human liver, skin, and kidneys (33, 34). The data of this study comparing CS and non-CS osmolytes indicate that the principle of cytoprotection is the underlying mechanism of the beneficial effects of ectoine.

Alternatively, nanoparticle-induced generation of reactive oxygen species has been proposed as one potential trigger of nanoparticle-induced MAPK signaling (35), and there is evidence that at least some CS possess antioxidant properties. However, it is accepted that ectoine cannot serve as an antioxidant (7). Our findings that nanoparticle stress elicits membrane dependent

signaling pathways involving epidermal growth factor receptor and integrins (17) suggest a mechanism involving the stabilization of macromolecules located at the outer cell surface. This hypothesis is further supported by the finding that ectoine is able to reduce pathogenic endpoints in skin epithelial cells induced by solar UVA radiation as a source for physical stress (14). Our studies demonstrated that this effect is due to a stabilization of lipid microdomains (rafts) in the cytoplasmic membrane and a decrease in UVA-induced ceramide release in human keratinocytes (15). Further studies will therefore address the role of raft signaling in CNP-induced stress responses in lung epithelial cells.

Conflict of Interest Statement: U.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. I.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.K. serves as a consultant to bitop AG; since July 2008, the Environmental Health Research Institute (IUF) has received a research grant from bitop for analysis of the mechanism of action of ectoine in lung epithelium. K.U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Petra Groß and Sigrid Martin for help in animal care and Ragnhild Wirth and Winfried Brock for excellent technical assistance.

References

- Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, MacNee W, Stone V. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. *Part Fibre Toxicol* 2005;2:10.
- Tao F, Gonzalez-Flecha B, Kobzik L. Reactive oxygen species in pulmonary inflammation by ambient particulates. *Free Radic Biol Med* 2003;35:327-340.
- MacNee W. Pathogenesis of chronic obstructive pulmonary disease. *Clin Chest Med* 2007;28:479-513.
- Bringardner BD, Baran CP, Eubank TD, Marsh CB. The role of inflammation in the pathogenesis of idiopathic pulmonary fibrosis. *Antioxid Redox Signal* 2008;10:287-302.
- Peters A, von Klot S, Heier M, Trentinaglia I, Hörmann A, Wichmann HE, Löwel H, Cooperative Health Research in the Region of Augsburg Study Group. Exposure to traffic and the onset of myocardial infarction. *N Engl J Med* 2004;351:1721-1730.
- Pandya RJ, Solomon G, Kinner A, Balmes JR. Diesel exhaust and asthma: hypotheses and molecular mechanisms of action. *Environ Health Perspect* 2002;110:103-112.
- Yancey PH. Organic osmolytes as compatible, metabolic and counter-acting cytoprotectants in high osmolarity and other stresses. *J Exp Biol* 2005;208:2819-2830.
- Barua M, Liu Y, Quinn MR. Taurine chloramine inhibits inducible nitric oxide synthase and TNF- α gene expression in activated alveolar macrophages: decreased NF- κ B activation and I κ B kinase activity. *J Immunol* 2001;167:275-281.
- Gurujeyalakshmi G, Hollinger MA, Giri SN. Regulation of transforming growth factor- β 1 mRNA expression by taurine and niacin in the bleomycin hamster model of lung fibrosis. *Am J Respir Cell Mol Biol* 1998;18:334-342.
- Schuller-Levis GB, Park E. Taurine and its chloramine: modulators of immunity. *Neurochem Res* 2004;29:117-126.
- Galinski EA, Pfeiffer H-P, Trüper HG. 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid: a novel cyclic amino acid from halophilic bacteria of the genus *Ectothiorhodospira*. *Eur J Biochem* 1985;149:135-139.
- Sauer T, Galinski EA. Bacterial milking: a novel bioprocess for production of compatible solutes. *Biotechnol Bioeng* 1998;57:306-313.
- Kanopathipillai M, Lentzen G, Sierks M, Park CB. Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer's β -amyloid. *FEBS Lett* 2005;579:4775-4780.
- Buenger J, Driller H. Ectoin: an effective natural substance to prevent UVA-induced premature photoaging. *Skin Pharmacol Physiol* 2004;17:232-237.
- Grether-Beck S, Timmer A, Felsner I, Brenden H, Brammertz D, Krutmann J. Ultraviolet A-induced signaling involves a ceramide-

- mediated autocrine loop leading to ceramide de novo synthesis. *J Invest Dermatol* 2005;125:545-553.
16. Sydlik U, Bierhals K, Soufi M, Abel J, Schins RP, Unfried K. Ultrafine carbon particles induce apoptosis and proliferation in rat lung epithelial cells via specific signaling pathways both using EGF-R. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L725-L733.
 17. Unfried K, Sydlik U, Bierhals K, Weissenberg A, Abel J. Carbon nanoparticle-induced lung epithelial cell proliferation is mediated by receptor-dependent AKT activation. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L358-L367.
 18. Albrecht C, Born PJ, Adolf B, Timblin CR, Mossman BT. *In vitro* and *in vivo* activation of extracellular signal-regulated kinases by coal dusts and quartz silica. *Toxicol Appl Pharmacol* 2002;184:37-45.
 19. Yamasawa H, Ishii Y, Kitamura S. Cytokine-induced neutrophil chemoattractant in a rat model of lipopolysaccharide-induced acute lung injury. *Inflammation* 1999;23:263-274.
 20. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1991;249:1431-1433.
 21. Zhang Q, Kleeberger SR, Reddy SP. DEP-induced fra-1 expression correlates with a distinct activation of AP-1 dependent gene transcription in the lung. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L427-L436.
 22. Gruenert DC, Willems M, Cassiman JJ, Frizzell RA. Established cell lines used in cystic fibrosis research. *J Cyst Fibros* 2004;3:191-196.
 23. Santos H, da Costa MS. Compatible solutes of organisms that live in hot saline environments. *Environ Microbiol* 2002;4:501-509.
 24. Driscoll KE, Carter JM, Iype PT, Kumari HL, Crosby LL, Aardema MJ, Isfort RJ, Cody D, Chestnut MH, Burns JL. Establishment of immortalized alveolar type II epithelial cell lines from adult rats. *In Vitro Cell Dev Biol Anim* 1995;31:516-527.
 25. Behndig AF, Mudway IS, Brown JL, Stenfors N, Helleday R, Duggan ST, Wilson SJ, Boman C, Cassee FR, Frew AJ, et al. Airway antioxidant and inflammatory responses to diesel-exhaust-exposure in healthy humans. *Eur Respir J* 2006;27:359-365.
 26. Sullivan DE, Ferris MB, Pociask D, Brody AR. Tumor necrosis factor- α induces transforming growth factor- β 1 expression in lung fibroblasts through the extracellular signal-regulated kinase pathway. *Am J Respir Cell Mol Biol* 2005;32:342-349.
 27. Antoniou KM, Ferdoutsis E, Bouros D. Interferons and their application in the diseases of the lung. *Chest* 2003;123:209-216.
 28. Wang P, Wu P, Anthes JC, Siegel MI, Egan RW, Billah MM. Interleukin-10 inhibits interleukin-8 production in human neutrophils. *Blood* 1994;83:2678-2683.
 29. Kim Y-M, Reed W, Lenz AG, Jaspers I, Silbejoris R, Nick HS, Samet JM. Ultrafine carbon particles induce interleukin-8 gene transcription and p38 MAPK activation in normal human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2005;288:432-441.
 30. Arakawa T, Timasheff SN. The stabilization of proteins by osmolytes. *Biophys J* 1985;47:411-414.
 31. Welch WJ, Brown CR. Influence of molecular and chemical chaperones on protein folding. *Cell Stress Chaperones* 1996;1:109-115.
 32. Lentzen G, Schwarz T. Extremolytes: natural compounds from extremophiles for versatile applications. *Appl Microbiol Biotechnol* 2006;72:623-634.
 33. Wettstein M, Häussinger D. Cytoprotection by the osmolytes betaine and taurine in ischemia-reoxygenation injury in the perfused rat liver. *Hepatology* 1997;26:1560-1566.
 34. Rockel N, Esser C, Grether-Beck S, Warskulat U, Flögel U, Schwarz A, Schwarz T, Yarosh D, Häussinger D, Krutmann J. The osmolyte taurine protects against ultraviolet B radiation-induced immunosuppression. *J Immunol* 2007;179:3604-3612.
 35. Unfried K, Albrecht C, Klotz L-O, von Mücke A, Grether-Beck S, Schins RPF. Cellular responses to nanoparticles: target structures and mechanisms. *Nanotoxicology* 2007;1:1-20.